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Antigenicity of *Campylobacter jejuni* Flagella

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We studied the antigenicity of a wild-type flagellate and motile (F^+M^+) *Campylobacter jejuni* strain (81116) and two daughter mutants, one flagellate and immotile (F^+M^-) and one aflagellate and immotile (F^-M^-). By sodium dodecyl sulfate-polyacrylamide gel electrophoresis of acid-extracted surface proteins, a 63-kilodalton (kDa) band identified from sheared flagella as the flagellar protein was present in the F^+M^+ and F^+M^- strains but not in the F^-M^- strain. No other differences in protein profile among the three strains were noted. By Western blotting, serum from rabbits immunized with either the F^+M^+ or F^-M^- strain detected a 63-kDa protein in the F^+M^+ and F^+M^- strains but not in the F^-M^- strain. That the F^-M^- antiserum recognized the 63-kDa band suggests that small amounts of this protein or a cross-reacting antigen is present on the F^-M^- strain. By counterimmunoelectrophoresis of the acid-extracted preparations with immune sera, all three strains were found to share three major antigens, but a fourth antigen with a net positive charge was present only in the F^+M^+ and F^+M^- strains. Antisera to five *C. jejuni* and two *Campylobacter fetus* strains recognized the 63-kDa protein of purified F^+M^+ flagella in Western blots, demonstrating that a common antigen is present, but enzyme-linked immunosorbent assay results suggest that the sharing of this antigen among *Campylobacter* strains is variable.

Campylobacter jejuni and closely related organisms are important pathogens causing diarrhea in humans (6, 18, 23). Although the pathogenesis of human infections with these organisms are not completely understood, the clinical characteristics of bloody diarrhea, cellular infiltration in the lamina propria, fever, and bacteremia suggest that *Campylobacter* species are invasive organisms (6). For other invasive organisms, vaccine development has been based on immunizing susceptible hosts with a surface component of the organisms. Several studies have indicated that a variety of *Campylobacter* species outer membrane proteins are antigenic for humans and other mammalian hosts (4, 13, 27, 22). Among those of interest are the flagellar proteins which are antigenic, and which are present on most *C. jejuni* strains. The development of aflagellate mutants and immotile flagellate mutants (17) enabled us to examine more closely the antigenicity of flagellar proteins for use as possible vaccines.

In the present report, we analyzed the structural, antigenic, and virulence characteristics of a flagellate motile (F^+M^+) parent strain 81116, a flagellate immotile (F^+M^-) daughter strain (F^+M^-), and an aflagellate immotile (F^-M^-) daughter strain. Purified flagella from the F^+M^+ strain appeared to contain a group antigen which was recognized by immune antisera produced against homologous and some heterologous *Campylobacter* strains.

MATERIALS AND METHODS

Bacterial strains. The wild-type strain (*C. jejuni* 81116 F^+M^+) was isolated from the stools of a patient with diarrhea (17) on Skirrow antibiotic selective medium (23) at 43°C under microaerobic conditions. The selection of immotile variants of *C. jejuni* has been described previously (17). Briefly, immotile organisms were enriched in the population by sequential subculture from the center of semisolid nutrient gelatin agar (1% peptone, 0.33% yeast extract, 0.5% sodium chloride, 0.8% gelatin-0.75% agar) stab cultures. Colonies of immotile organisms were identified by dark-field microscopy and by the type of growth on nutrient agar plates with 1% agar (motility agar) and blotted onto nitrocellulose paper. Incubation of the wet blots in methanol containing 0.5% hydrogen peroxide eliminated endogenous peroxidase. Nonspecific binding was eliminated by incubation of the nitrocellulose blots in 3% bovine serum albumin in 10 mM Tris hydrochloride buffer (pH 7.4) in 0.9% sodium chloride for 2 h at 37°C. Flagellate and aflagellate colonies were differentiated by incubation for 2 h at 37°C in a 1:100 dilution of rabbit anti-flagellum antiserum (17) followed by incubation in a 1:1,000 dilution of peroxidase-conjugated goat anti-rabbit immunoglobulin G antiserum (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.). Antisera were diluted in 50 mM Tris hydrochloride containing 150 mM sodium chloride, 5 mM EDTA, 0.25% gelatin, and 0.05% Nonidet P-40. Unbound protein was removed by extensive washing in 500 mM Tris hydrochloride containing 1 M sodium chloride, 50 mM EDTA, 0.25% gelatin, and 0.4% sodium lauryl sarcosinate, and peroxidase was detected by the *O*-dianisidine substrate. Approximately 4% of the immotile colonies in the enriched population were flagellate. The wild-type strain and the variants were cloned six times to confirm stability. The wild-type strain and the two variants were biotyped by the method of Skirrow and Benjamin (24) and serotyped by the passive hemagglutination technique of Penner and Hennessy (19). Flagellar staining was performed by the method of Kodaka et al. (12). For comparative studies we used *C. jejuni* 79-193, 84-25, PEN 1, and PEN 2 and *C. fetus* 23D (84-32), 82-40, and 81-170, for which previous antigenic analyses have been described (3, 4, 20, 21).

Preparation of flagella. Isolated flagella were prepared by a modification of the method of Dipamphilis and Adler (9).

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Campylobacter strains were grown on Mueller-Hinton plates with 5% sheep blood (PASCO, Wheat Ridge, Colo.) for 24 h at 37 or 42°C in a microaerobic atmosphere. After cells were harvested in sterile distilled water and centrifuged, the pellet was suspended in Tris (0.1 M) to an optical density at 450 nm of 1.5. The cell suspension was sheared by a Virtis blender for 45 to 60 s at 0°C, diluted sixfold in Tris, and centrifuged at 12,000 × *g* for 10 min to pellet cell debris. The supernatant then was centrifuged at 55,000 ×*g* for 60 min to sediment sheared flagella. Each pellet was resuspended overnight at 4°C in Tris, and the 12,000 and 55,000 × *g* sedimentations were repeated. Protein and 2-keto-3-deoxyoctulosonic acid concentrations in the final pellets were determined as described previously (3). Electron microscopy of whole *C. jejuni* cells and purified flagella was based on the method of Montie et al. (16). Samples of the flagellum preparations (10 μl) were placed on Formvar-coated carbon-stabilized 200-mesh copper grids (Ted Pella, Tustin, Calif.). After the grids were air dried, they were stained for 1 min in 1% phosphotungstic acid (pH 7.0), rinsed with distilled water, and allowed to air dry. The grids then were examined by a Phillips 300 electron microscope at 60 kV accelerating voltage.

SDS-PAGE. Analysis of cellular proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done as described previously (3). Fractions studied included whole cells, sarcosyl-insoluble (outer) membranes (3), blebs (3), and acid-extracted surface material (15, 22) prepared as described previously (2). In brief, aliquots standardized with approximately 1.0 μg of protein (14) in the sample buffer were loaded into 4.5% stacking gels, and 8 to 12% separating gels were used. Electrophoresis was carried out at 35 mA for 2 h, gels were then fixed, and proteins were resolved by silver stain.

Immunoblot procedure. The Western blot procedure we used was as described previously (4). After SDS-PAGE, proteins were electroblotted at 100 mA for 18 h onto nitrocellulose paper soaked in buffer. The nitrocellulose paper was preincubated with a solution containing 1% bovine serum albumin and washed, and a 1:1,000 dilution of test sera was applied. After incubation and washing, the nitrocellulose paper was incubated with a 1:1,000 dilution of horseradish peroxidase-conjugated *Staphylococcus aureus* protein A, and color was developed as previously described (4). Immune rabbit sera to whole F^+M^+, F^+M^-, and F^-M^- cells, and cells of several other *Campylobacter* strains were obtained by methods described previously (5). Certain sera were absorbed with live *C. jejuni* cells before use in the immunoblot studies. Growth from one plate was suspended in distilled water, bacterial cells were collected by centrifugation at 12,000 × *g* for 15 min, and the pellet was mixed with 1.0 ml of rabbit serum and incubated at 37°C for 1 h. The bacterial suspension was removed by centrifugation at 12,000 × *g* for 15 min, and the antibody-containing supernatant was reabsorbed three times.

ELISA. The enzyme-linked immunosorbent assay (ELISA) used in this study was similar to a previous assay (21). In brief, purified flagella from each of eight strains (*C. jejuni* F^+M^+, F^+M^-, 79-193, 84-25, PEN 2, and PEN 3, and *C. fetus* 81-170 and 84-32 [23D]) were diluted in carbonate buffer (pH 9.6) to a final protein concentration of 1 μg/ml, and 0.2 ml was used to coat wells of polystyrene microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.). Twofold serial dilutions of antiserum raised against purified flagella to the F^+M^+ strain as described before (17) and normal rabbit serum in 5% bovine gamma globulin–0.1% gelatin represented the first antibodies, and a 1:2,000 dilution of peroxidase-conjugated swine anti-rabbit immunoglobulin (Accurate Chemicals, Westbury, N.Y.) represented the detection antibody. The developing solution consisted of 2,2'-azino-di-(3-ethyl-benzthiasoline sulfonic acid) in McIlvain buffer with hydrogen peroxide. All assays were performed in triplicate, and results were read on a Titertek Multiscan (Dynatech) at 414 nm, as previously described (2).

Counterimmunoelectrophoresis. Counterimmunoelectrophoresis of acid-extracted surface material and purified flagella was done by the method of Crowle (8). In brief, 5 μl of immune antisera and antigens to be studied were applied to the anodic and cathodic wells, respectively, of a 1.4% agarose–0.1 M barbital buffer (pH 8.6) gel. After electrophoresis at 40 mA for 30 min, gels were kept at 4°C overnight, washed with agitation in 0.01 M phosphate buffered saline (pH 7.4) for 48 h with buffer changes every 12 h, and followed by a 4-h wash in distilled water. After the final wash, gels were stained in Crowle double stain (10) for 30 min, and then differentiated in 0.3% acetic acid.

RESULTS

Characterization of the variants. The biochemical analysis of the wild-type strain and the variants showed that all were catalase and oxidase positive and sensitive to naladixic acid; they all hydrolyzed hippurate, produced hydrogen sulfide, and were serotype PEN 6. The colonies of the wild-type strain were spreading, while those of the variants were compact. By dark-field microscopy, the wild-type organisms had a characteristic darting motility, but the organisms of both of the variants were immotile. Both flagellar staining

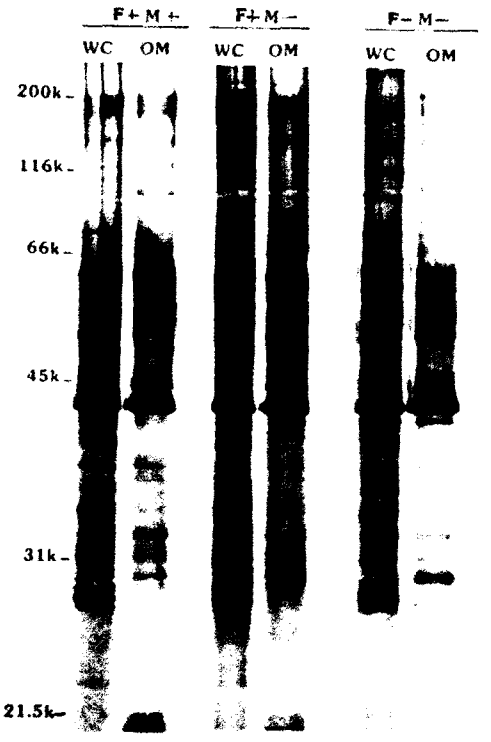

FIG. 1. SDS-PAGE (10% acrylamide) profile of whole cell (WC) and outer membrane (OM) by sarcosyl extraction of the three related *C. jejuni* strains. Molecular weights of marker proteins are indicated at left. The F^-M^- strain does not include the major band seen in the 65 kDa region present in similar preparations of the other two strains.

and electron microscopy confirmed that the F⁺M⁺ and F⁺M⁻ strains were flagellate, whereas the F⁻M⁻ strain was not.

SDS-PAGE profiles of the three strains. The whole-cell and outer membrane SDS-PAGE profiles were nearly identical for the F⁺M⁺, F⁺M⁻, and F⁻M⁻ strains (Fig. 1). The major band resolved in all three preparations migrated at 43 kilodaltons (kDa) as has been identified for other *C. jejuni* strains (3, 13), with numerous other minor bands resolved in this gel. However, the F⁺M⁺ and F⁺M⁻ strains showed bands migrating at about 63 kDa which were not present in the F⁻M⁻ strain. Acid-extracted surface protein preparations contained bands at 31 and 29 kDa for all three strains, but a 63-kDa band was present only for the F⁺M⁺ and F⁺M⁻ strains (data not shown).

Properties of purified flagella. From six 150-mm plates with confluent growth of the F⁺M⁺ strain, the shearing procedure yielded 0.945 mg of flagellar protein; no 2-keto-3-deoxy-octulosonic acid was detected. By electron microscopy, a homogeneous preparation containing numerous flagellar filaments and little debris was seen with negative staining (Fig. 2). SDS-PAGE of isolated flagella after the shearing procedure showed that a major band migrated at about 63-kDa, and two more minor bands were resolved at 92 and 43 kDa (Fig. 3). The latter band represents the major outer membrane protein (porin) of *C. jejuni* strains (3, 13). This gel showed that the specific band not present in the F⁻M⁻ strain migrated at the same molecular weight as that of the purified flagellar protein.

FIG. 2. Electron microscopy of sheared flagella from the F⁺M⁺ strain of 81116. Magnification, ×32,000. Bar, 1 μm.

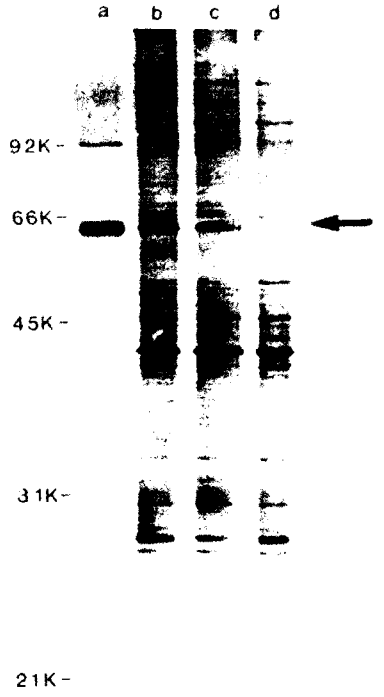

FIG. 3. SDS-PAGE (10% acrylamide) of isolated flagella from F⁺M⁺ (lane a), and whole cells of F⁺M⁺ (lane b), F⁺M⁻ (lane c) and F⁻M⁻ (lane d). The arrow indicates that the 63-kDa band that the F⁻M⁻ strain lacks and that is seen in the other two strains has the same migration as the major protein in the flagellar preparation.

Antigens identified by Western blot analysis. We used both the whole-cell and the acid-extracted surface material of the three strains for Western blot analysis of antigens. Normal rabbit serum did not react with any portion of the SDS-PAGE preparations electroblotted onto nitrocellulose paper (data not shown). In contrast, serum from a rabbit immunized with the F⁺M⁺ strain recognized a large number of bands in the whole-cell preparations (Fig. 4). The bands recognized for each of the three strains were virtually identical, except that at about 63 kDa, the F⁺M⁺ and the F⁺M⁻ strains showed doublet bands, whereas the F⁻M⁻ strains showed only a singlet. This difference was even more marked in the acid-extracted materials. The homologous serum strongly reacted to the 63-kDa region of the F⁺M⁺ strain and reacted slightly less to the F⁺M⁻ strain, but recognition of that region on the F⁻M⁻ strain was minimal. This was the only difference in the recognition of the acid-extracted preparations from the three strains. Results of Western blotting the same materials with serum from a rabbit immunized with the F⁻M⁻ strain were nearly the same. In the whole-cell preparations, the F⁻M⁻ antiserum recognized the doublet migrating at about 63 kDa for the F⁺M⁺ and F⁺M⁻ strains but only a singlet for the homologous F⁻M⁻ strain (data not shown). Again, recognition of that region in the acid-extracted surface preparations was much enhanced for the F⁺M⁺ and F⁺M⁻ strains compared with the F⁻M⁻ strain.

Antigenicity of purified flagellae. The predominant antigen in the sheared F⁺M⁺ flagellar preparation was the flagellar protein (Fig. 5). This band was recognized by the homolo-

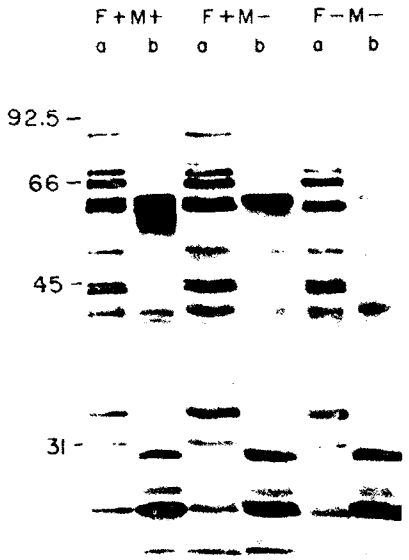

FIG. 4. Western blot of whole cell (lanes a) and acid-extracted surface material from 12% acrylamide gel (lanes b) of three related *C. jejuni* strains (F^+M^+, F^+M^-, and F^-M^-, as described in the legend to Figure 1) with antiserum to the F^+M^+ strain. F^+M^+-immune rabbit serum and normal rabbit serum (results not shown) were diluted 1:100, and the capture antibody was horseradish peroxidase-conjugated staphylococcal protein A.

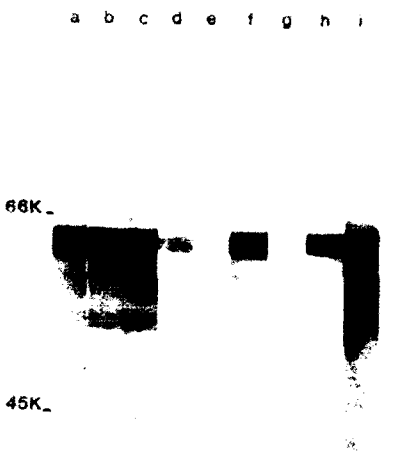

FIG. 5. Western blot of sheared F^+M^+ flagella from 10% acrylamide gel with *Campylobacter* spp. immune serum. The flagellar protein preparation was loaded (1 μg/5 mm of SDS-PAGE horizontal dimension), and immunoblotting was performed as described in the text. Sera were diluted 1:100. Sera in lanes were raised against: a, F^+M^+; b, F^+M^-; c, F^-M^-; d, *C. jejuni* PEN 1; e, PEN 1, then absorbed with F^+M^+ cells; f, *C. jejuni* PEN 2; g, normal rabbit serum; h, *C. fetus* 82-40; and i, *C. fetus* 81-170.

gous F^+M^+, F^+M^-, and F^-M^- antisera, two heterologous *C. jejuni* antisera, two *C. fetus* antisera but not by normal rabbit serum. Absorption of one heterologous antiserum with F^+M^+ whole cells (Fig. 5, lane e) eliminated the reactivity of the antiserum. To compare antigenicity of isolated flagella from other strains, we immunoblotted the preparations with unabsorbed and absorbed homologous antisera raised to the whole bacterial cells (Fig. 6). These blots again indicated the relative purity of sheared flagellar preparations, with only the major outer membrane protein being present in small amounts in some preparations. For each of the four preparations, the homologous antiserum recognized the flagellar protein, but this activity was largely ablated by absorption of the serum with homologous cells. Absorbing the antiserum to the F^+M^+ strain with F^-M^- cells partially removed antibody specific for the flagellar band, but absorption with PEN 2 cells removed little. Conversely, absorption of PEN 2 antiserum with either the F^+M^+ or F^-M^- cells removed most of the activity. Absorbing antiserum to *C. fetus* strain 23D with *C. jejuni* cells had a partial effect on residual antiflagellar antibody.

These results were confirmed by an ELISA which provided more quantitative data (Fig. 7). In this assay, antiserum to the F^+M^+ flagellae recognized the F^+M^- flagella essentially to the same extent as it did for the homologous F^+M^+ preparation. In contrast, there was little recognition of the flagella from the PEN 2, PEN 3, or 81-170 strains. Recognition of flagella from two other *C. jejuni* strains, 79-193 and 84-25, and *C. fetus* 23D were intermediate. A repeat assay showed nearly identical results (data not shown).

Counterimmunoelectrophoresis of acid-extracted materials. We studied the antigens expressed in acid-extracted materials from the three related strains and in purified F^+M^+ flagella as recognized by antisera to the F^+M^+ strain (Fig. 8). Although quantitative differences were present, each strain had one dominant positively charged antigen recognized in the acid-extracted materials, and two other positively charged antigens were present in the F^+M^+ and F^+M^- strains only. The F^+M^+ antiserum also recognized in the F^+M^+ and F^+M^- strains, but not in the F^-M^- strain, a negatively charged antigen migrating toward the positive

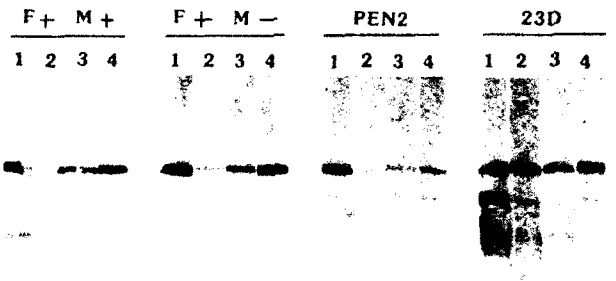

FIG. 6. Western blots of purified *Campylobacter* flagella with unabsorbed and absorbed homologous antisera. Flagellar preparations are from (left to right) F^+M^+, F^+M^-, PEN 2 *C. jejuni* strains, and 23D *C. fetus* strain. For each preparation, lane 1 represents the unabsorbed antiserum raised to whole homologous cells, whereas lanes 2 through 4 represent that antiserum absorbed with various cells. Absorptions of the antisera to F^+M^+, F^+M^-, and 23D are with F^+M^+ (lanes 2), F^-M^- cells (lanes 3), and PEN 2 cells (lanes 4). Absorptions of PEN 2 antiserum are with PEN 2 cells (lane 2), F^+M^+ cells (lane 3), and F^-M^- cells (lane 4).

pole. The purified F⁺M⁺ flagella showed a single major negatively charged antigen.

DISCUSSION

The use of F⁻M⁻ mutants of F⁺M⁺ *C. jejuni* strains is a potentially powerful technique for assessing the role of flagella in antigenicity of *C. jejuni*. By both electron and light microscopy, flagella were clearly seen on the F⁺M⁺ and F⁺M⁻ strains but not on the F⁻M⁻ strain. The F⁻M⁻ and F⁻M⁻ strains are phenotypically similar to the parent F⁺M⁺ strain by several biochemical criteria, but examination of colonies on agar showed a direct correlation of motility and colony size. Genetic analysis has not been done, but differences in antigenic characteristics might be due to deletion or switching of genes not related to flagellar structure and function. Immotile or aflagellate mutants of flagellate parent strains that are clearly isogenic will be needed to definitively answer such questions.

Confirming an earlier analysis (17), our SDS-PAGE studies clearly showed that the F⁻M⁻ strain is missing a 63-kDa protein, which in the other strains represents a major band that is outer membrane associated and in our studies is acid extractable from the cell surface. Purification of flagella from the F⁺M⁺ strain demonstrated that the major flagellar protein migrated at about 63 kDa, confirming that the aflagellate strain is lacking this specific protein. Furthermore, Western blot analysis showed that both the F⁺M⁺ and F⁺M⁻ strains possessed an antigenically similar or identical band migrating at 63 kDa, whereas it was not present on the F⁻M⁻ strain. Wenman and colleagues working with the same F⁻M⁻ strain also noted that this strain lacks a 63-kDa band, but that a spontaneous revertant once again acquires that band (25).

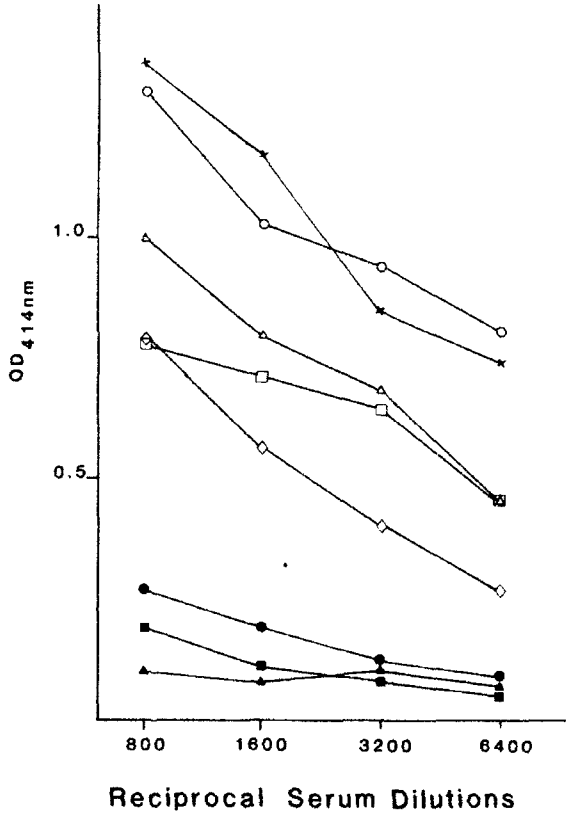

FIG. 7. Recognition of purified flagella from various *Campylobacter* strains by antiserum to flagella of *C. jejuni* strain F⁺M⁺ by ELISA. Antiserum is from a rabbit hyperimmunized with F⁺M⁺ flagella as described, and the second antibody is horseradish-peroxidase conjugated swine anti-rabbit immunoglobulin. Flagellar preparations were fixed to microtiter wells (0.2 μg per well) and were from F⁺M⁺ (○), F⁺M⁻ (X), 23D (△), 79-193 (□), 84-25 (◇), PEN 2 (●), 81-170 (▲), and PEN 3 (■). Each point represents the mean of triplicate determinations.

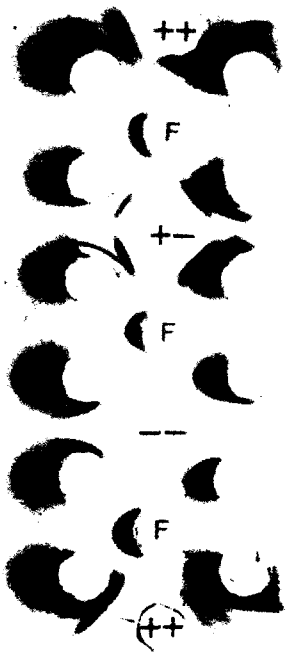

FIG. 8. Counterimmunoelectrophoresis of sheared F⁺M⁺ flagella (F), or acid-extracted material from F⁺M⁺ (++), F⁺M⁻ (+−), and F⁻M⁻ (−−) *C. jejuni* strains with F⁺M⁺-immune rabbit serum. The antigen-containing wells are in the center with the F⁺M⁺ antibody-containing wells on the sides. The anode is on the right, and the cathode is on the left.

However, this same band is recognized by immune serum raised against the F⁻M⁻ strain, indicating that this organism contains similar or identical antigenic determinants that were not resolved at 63 kDa by the SDS-PAGE and immunoblot techniques. One possibility is that the 63 kDa flagellar protein is a polymer, whereas the F⁻M⁻ strain has monomeric or other smaller units present on its surface. Another possibility is that the F⁻M⁻ strain possesses the intact 63 kDa protein on its surface in quantities too small to detect by either technique but sufficiently immunogenic in rabbits to elicit a response in vivo. A third possibility is that a small number of the F⁻M⁻ strains used for vaccinating the rabbits reverted during the in vitro growth conditions to flagellate forms. Recently, Caldwell and colleagues (7) found that the F⁻M⁻ strain converted back to a flagellate form (F⁺M⁺) at a rate of 2.1×10^{-6} per cell generation in vitro. Even more striking was their observation that two and four days after feeding the F⁻M⁻ strain to surgically manipulated rabbits, only F⁺M⁺ forms were recovered from their feces. These data support the hypothesis of spontaneous reversion of some F⁻M⁻ cells before they were used for immunizing rabbits; however, whether the small number of revertants that could be anticipated were sufficient to elicit an antibody response to the flagellar protein is unknown. Further studies of this phenomenon are warranted.

By use of counterimmunoelectrophoresis techniques, the acid-extracted surface material of F^+M^+ and F^+M^- strains was shown to possess several major antigens with either net negative or net positive charge. The acid-extracted material of the F^-M^- strain lacked antigens with net positive or net negative charges, charges that the F^+M^+ and F^+M^- strains possessed. Use of the purified flagella indicated that the major flagellar antigen had a net negative charge. By studying acid-extracted material of other *C. jejuni* strains by counterimmunoelectrophoresis (M. J. Blaser and H. J. Cody, unpublished data), it was clear that similarly migrating antigens were present on each of these flagellate strains, suggesting that species-specific common flagellar antigen(s) may be present, extending the observations of Rautelin and Kosunen (22).

Studies with the purified flagellar preparation demonstrated that the protein(s) migrating at about 63 kDa were recognized to different degrees by antisera to a variety of *C. jejuni* and *C. fetus* strains. Whereas our earlier studies showed that the major outer membrane (porin) proteins of *C. jejuni* and *C. fetus* are not antigenically cross-reactive (4), the present data suggest that the flagellar proteins might be cross-reactive. The use of purified flagellar preparations from a variety of strains and antiserum to the purified F^+M^+ flagella in an ELISA confirmed the presence of a group antigen(s) in the F^+M^+ flagella that cross-reacted with other *Campylobacter* flagella, although cross-reactivity appeared to be variable. In another study of immunoblots of *C. fetus* outer membranes with *C. jejuni* antisera, recognition of the flagellar band was poor for three *C. fetus* strains but excellent for another (25).

Our studies suggest that further examination of the flagellar proteins of *C. jejuni* will be a useful endeavor. Wenman and colleagues (25) found that the *C. jejuni* flagellar protein is an essential determinant of the heat-labile antigen-typing scheme and is a dominant antigen recognized during *C. jejuni* infections in humans. As with investigations of other organisms, the definition of common flagellar antigens may be of value for taxonomic and diagnostic purposes, development of a subunit type vaccine, and study of virulence mechanisms (1, 11).

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